



Novel Alphacoronaviruses and Paramyxoviruses Cocirculate with Type 1 and Severe Acute Respiratory System (SARS)-Related Betacoronaviruses in Synanthropic Bats of Luxembourg

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ABSTRACT Several infectious disease outbreaks with high mortality in humans have been attributed to viruses that are thought to have evolved from bat viruses. In this study from Luxembourg, the genetic diversity and epidemiology of paramyxoviruses and coronaviruses shed by the bat species *Rhinolophus ferrumequinum* and *Myotis emarginatus* were evaluated. Feces collection ($n = 624$) was performed longitudinally in a mixed-species colony in 2015 and 2016. In addition, feces ($n = 254$) were collected cross-sectionally from six *Myotis emarginatus* colonies in 2016. By use of degenerate primers in a nested format, overall prevalences of 1.1% (10/878) and 4.9% (43/878) were determined for paramyxoviruses and coronaviruses. Sequences of the partial RNA-dependent RNA polymerase and spike glycoprotein genes of coronaviruses, as well as sequences of the partial L gene of paramyxoviruses, were obtained. Novel paramyxovirus and Alphacoronavirus strains were identified in different *Myotis emarginatus* colonies, and severe acute respiratory syndrome (SARS)-related Betacoronavirus strains were shed by *Rhinolophus ferrumequinum*. Logistic regression revealed that the level of Alphacoronavirus shedding was highest in July (odds ratio, 2.8; $P < 0.01$), probably due to periparturient stress. Phylogenetic analyses point to close virus-host coevolution, and the high genetic similarity of the study strains suggests that the *Myotis emarginatus* colonies in Luxembourg are socially connected. Most interestingly, we show that bats also host Betacoronavirus 1 strains. The high similarity of the spike gene sequences of these viruses with mammalian Betacoronavirus 1 strains may be of concern. Both the SARS-related and Betacoronavirus 1 strains detected in bats in Luxembourg may cross the species barrier after a host adaptation process.

IMPORTANCE Bats are a natural reservoir of a number of zoonotic pathogens. Several severe outbreaks in humans (e.g., a Nipah virus outbreak in Malaysia in 1998, and the almost global spread of severe acute respiratory syndrome in 2003) have been caused by bat-borne viruses that were transmitted to humans mostly after virus adaptation (e.g., in intermediate animal hosts). Despite the indigenoussness of bat species that host viruses with suspected zoonotic potential and despite the zoonotic transmission of European bat 1 lyssavirus in Luxembourg, knowledge about the diversity and epidemiology of bat viruses remains limited in this country. Moreover, in contrast to other European countries, bat viruses are currently not included in the national surveillance activities of this land-locked country. We suggest that this gap in disease surveillance should be addressed, since we show here that synanthropic bats host viruses that may be able to cross the species barrier.

KEYWORDS Chiroptera, Luxembourg, coronavirus, molecular epidemiology,

Received 14 June 2017 Accepted 5 July 2017

Accepted manuscript posted online 14 July 2017

Citation Pauly M, Pir JB, Loesch C, Sausy A, Snoeck CJ, Hübschen JM, Muller CP. 2017. Novel alphacoronaviruses and paramyxoviruses cocirculate with type 1 and severe acute respiratory system (SARS)-related betacoronaviruses in synanthropic bats of Luxembourg. Appl Environ Microbiol 83:e01326-17. <https://doi.org/10.1128/AEM.01326-17>.

Editor Edward G. Dudley, The Pennsylvania State University

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paramyxovirus, phylogenetic analysis, surveillance studies, virology, zoonotic infections

The ability of bats (Chiroptera) to fly long distances and their longevity enable them to spread viruses across time and space. Large colony sizes, close social interactions, and coroosting of different bat species favor intraspecies and interspecies transmission of viruses (1). Moreover, the low pathogenicity of viruses and their persistence in bats are indicative of ancient cospeciation between bats and different virus families (e.g., *Paramyxoviridae* and *Coronaviridae* [2–5]). It has been suggested that most human coronaviruses (CoV) evolved from bat counterparts (5–7). For instance, severe acute respiratory syndrome CoV (SARS-CoV) (8) and Middle East respiratory syndrome CoV (MERS-CoV) (9, 10), as well as the paramyxoviruses (PV) Nipah virus and Hendra virus (11, 12), originated in bats and have caused severe outbreaks in humans. While for some viruses, viral adaptation processes in intermediate animal hosts were presumably required before zoonotic transmission (see, e.g., references 9 and 13), direct transmission of Nipah virus between bats and humans has occurred repeatedly in Bangladesh (14). The spike glycoproteins of several bat CoV strains share features with human strains that have been critical for bat-to-human transmission events (15). In particular, the receptor-binding domain of the spike gene determines the host range and tissue tropism of CoV (16–18). Nevertheless, the risk of zoonotic infection with bat viruses is low for humans, since direct contacts with bat excretions are rare (19, 20). In addition, the risk can be monitored by virus surveillance in synanthropic bats (20), such as vespertilionid (e.g., *Myotis emarginatus*) and rhinolophid (e.g., *Rhinolophus ferrumequinum*) bats, which have been shown to host a number of viruses with zoonotic potential (5, 21–23).

In Western and Central Europe, *M. emarginatus* and *R. ferrumequinum* are endangered (24, 25) due to ongoing habitat fragmentation (26). After hibernating in underground sites, *R. ferrumequinum* females return to their natal colonies in March, while *M. emarginatus* females follow only in May (27–29). They form matrilineal maternity colonies in attics and barns (27–29). Around mid-June, each female gives birth to a single pup. Intralinear polygyny is common for *R. ferrumequinum* (30, 31), and extracolony mating of *R. ferrumequinum* and *M. emarginatus* bats occurs during the swarming of the males, between September and October (32, 33).

Despite a growing interest in these animals as hosts of emerging viruses, knowledge about bat viruses in Luxembourg remains limited. In a single study, European bat 1 lyssavirus was isolated and the risk of zoonotic transmission in the country shown (34).

Here we report the shedding of PV and CoV by *R. ferrumequinum* and *M. emarginatus*, two sympatric and synanthropic bat species. Virus diversity and prevalence were assessed in six nursing colonies of *M. emarginatus* in a cross-sectional manner. In addition, we investigated the seasonal patterns of both viruses in a mixed *R. ferrumequinum*–*M. emarginatus* colony, in a parallel longitudinal study. Several novel viruses of both families were detected, and we show that bats are also a host for *Betacoronavirus* 1 strains.

RESULTS

To assess the prevalence and diversity of PV and CoV shedding among bats in Luxembourg, fecal samples from 7 colonies (Fig. 1A) were screened using degenerate primers in a nested format. The overall prevalence of PV was 1.1% (10/878), and that of CoV was 4.9% (43/878); viruses were found in every colony except for those at Colpach and Marienthal (Fig. 1A; Table 1). No PV–CoV coinfections were detected.

Bat PV were detected only in Ettelbruck and Bech-Kleinmacher (Fig. 1A), and shedding rates never exceeded 0.8 to 3.6% throughout the observation period. Because of the low prevalence rates, statistical analyses of seasonal variation were not possible for PV. Nine of the 10 PV strains detected were nearly identical to each other (represented by LUX15-A-033 and LUX15-A-351 in Fig. 2). BLAST and phylo-

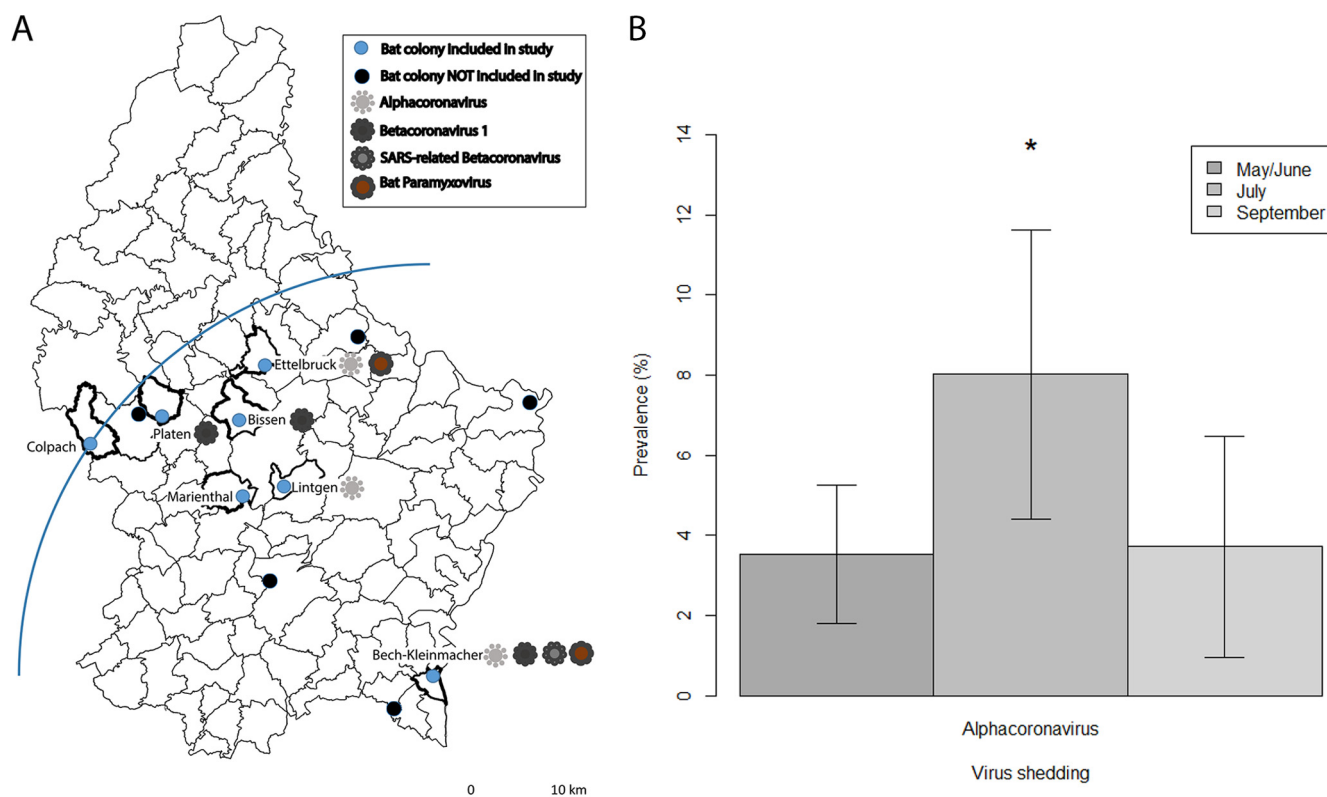


FIG 1 (A) Municipalities with known *Myotis emarginatus* colonies in Luxembourg and circulation of coronavirus and paramyxovirus strains. The blue quadrant with the mixed colony of Bech-Kleinmacher as the center has a radius of 45 km and includes all colonies investigated. The base map is from the Land Registry Office of the Grand Duchy of Luxembourg. (B) Seasonality of alphacoronavirus shedding in Bech-Kleinmacher. Error bars represent the 95% confidence interval; *, $P < 0.05$.

genetic analyses revealed that our PV strains were most closely related to those of insectivorous bats from China and South Africa, with which they shared <80% nucleotide identity and <92% amino acid identity. Based on the phylogenetic analyses (Fig. 2), all study sequences were grouped into a well-supported cluster, comprising also the unassigned murine J virus (25), Beilong virus (35), and other Jeilong virus-related PV (2, 3).

From the CoV strains detected in this study, partial RNA-dependent RNA polymerase (RdRp) gene sequences were obtained. We show that strains of 2 of the 4 currently recognized CoV genera (i.e., *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*) circulate in Luxembourg, and 36 *Alphacoronavirus* and 7 *Betacoronavirus* were detected (Fig. 3; Table 1). *M. emarginatus* bats from three different colonies (i.e., Ettelbruck, Lintgen, and Bech-Kleinmacher) (Fig. 1A; Table 1) shed nearly identical alphacoronaviruses (>99% nucleotide identity between partial RdRp gene sequences), most closely related to CoV circulating among insectivorous bats in China (Fig. 3). In contrast to the PV shedding, *Alphacoronavirus* shedding was variable in Bech-Kleinmacher; the highest rates were observed in July after parturition (odds ratio [OR], 2.8; $P < 0.01$) (Fig. 1B). *Alphacoronavirus* strains from this study (represented by LUX15-A-48 in Fig. 3) formed a distinct cluster, and their RdRp gene sequences shared <86% amino acid identity with other sequences classified as *Alphacoronavirus*.

On the phylogenetic tree of the partial RdRp gene, the *Betacoronavirus* strains from this study clustered within 2 of the 4 recognized lineages (A to D) of *Betacoronavirus* (<https://talk.ictvonline.org>): *R. ferrumequinum* bats shed SARS-related CoV (lineage B, represented by LUX16-A-24 in Fig. 3), and *M. emarginatus* bats shed *Betacoronavirus 1* (lineage A, represented by LUX15-A-158 in Fig. 3). The SARS-related CoV from Bech-Kleinmacher were identical to each other, and BLAST analyses revealed 94% nucleotide

TABLE 1 Characteristics of the different colonies and of the data set, as well as detection rates of coronaviruses and paramyxoviruses

Location of colony (UTM coord. ^a)	Bat species	Population size on 10/06/2016	Sample collection date (day/mo/yr)	No. of samples (% of population)	No. (%) of bat fecal samples in which nucleic acids of the following virus(es) were detected:				
					All CoV ^{b,c}	alphaCoV ^b	SARS-related CoV ^c	betaCoV ^b	Bat PV ^b
Lintgen (32U 293/5511)	<i>Myotis emarginatus</i>	65	10/06/2016	44 (67.7)	8 (18.2)	8 (18.2)	0 (0)	0 (0)	0 (0)
Ettelbruck (32U 291/5525)	<i>Myotis emarginatus</i>	220	10/06/2016	44 (20)	1 (2.3)	1 (2.3)	0 (0)	0 (0)	1 (2.3)
Marienthal (32U 288/5510)	<i>Myotis emarginatus</i>	45	03/06/2016	45 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Bissen (32U 288/5519)	<i>Myotis emarginatus</i>	35	03/06/2016	33 (94.3)	1 (3)	0 (0)	0 (0)	1 (3)	0 (0)
Colpach (31U 703/5515)	<i>Myotis emarginatus</i>	70	10/06/2016	44 (62.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Platen (31U 711/5519)	<i>Myotis emarginatus</i>	60	16/06/2016	44 (73.3)	2 (4.6)	0 (0)	0 (0)	2 (4.6)	0 (0)
Bech-Kleinmacher (32U 308/5489)	<i>Myotis emarginatus</i> and <i>Rhinolophus ferrumequinum</i>	942	09/06/2015	100 (10.6)	1 (1)	0 (0)	0 (0)	1 (1)	1 (1)
			14/07/2015	126 (13.4)	15 (11.9)	15 (11.9)	0 (0)	0 (0)	1 (0.8)
			04/09/2015	100 (10.6)	7 (7)	6 (6)	0 (0)	1 (1)	2 (2)
			17/05/2016	99 (10.5)	3 (3)	1 (1)	2 (2)	0 (0)	1 (1)
			11/07/2016	111 (11.8)	5 (4.5)	5 (4.5)	0 (0)	0 (0)	4 (3.6)
			09/09/2016	88 (9.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total		1,437		878 (61.1)	43 (4.9)	36 (4.1)	2 (0.2)	5 (0.6)	10 (1.1)

^aUTM coord., Universal Transverse Mercator coordinates.
^bbetaCoV, Betacoronavirus 1. Detected in *Myotis emarginatus*.
^cDetected in *Rhinolophus ferrumequinum*.

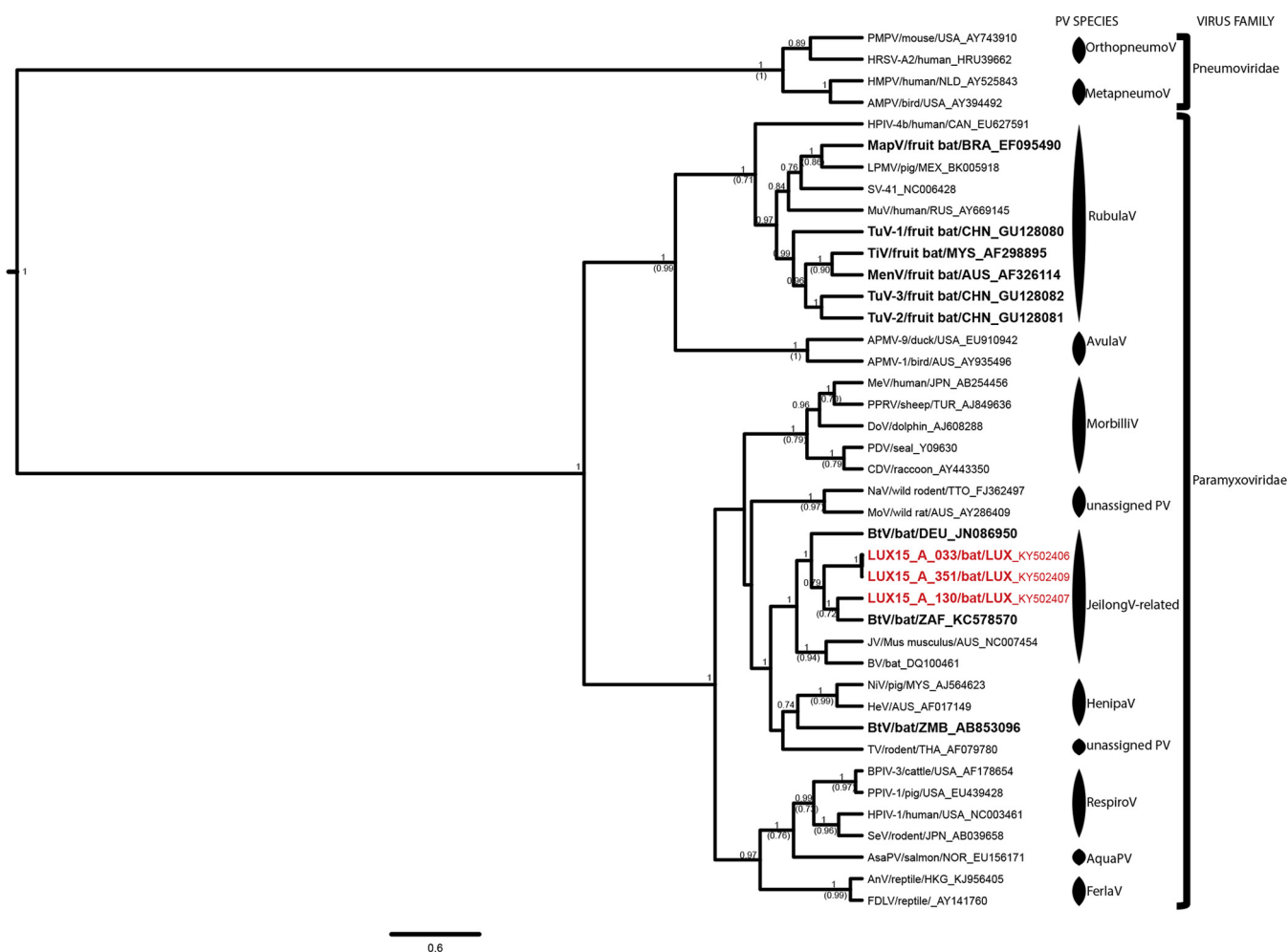


FIG 2 Phylogenetic analysis of the partial L genes of *Paramyxoviridae*. Shown are results of Bayesian analyses of a 410-nt alignment comprising the unique partial L gene sequences of 34 PV strains representing all PV species recognized by the International Committee on Taxonomy of Viruses, as well as novel unassigned but putative PV species. Three of the 10 PV from this study were added to the data set to represent the genetic diversity of PV circulating in *Myotis emarginatus* populations in Luxembourg. Four *Pneumoviridae* strains served as the outgroup for the phylogenetic analyses. The study sequences are shown in red, and strains hosted by bats are shown in boldface to reveal the high genetic diversity of bat PV. Only the pp values of well-supported nodes (pp > 0.7) are shown, and if the nodes were also supported by ML inference (bootstrap confidence levels above 0.7), the bootstrap support is shown in parentheses. For each cluster, the PV species, as well as the virus family assignment, are shown. The sequences were named, if the information was available, according to the following nomenclature: abbreviated virus name/host species/three-letter code of the country of origin_GenBank accession number. PMPV, pneumonia virus of mice; HRSV, human respiratory syncytial virus; HMPV, human metapneumovirus; AMPV, avian metapneumovirus; HPIV, human parainfluenza virus; MapV, Mapuera virus; LPMV, porcine rubulavirus; SV, simian virus; MuV, mumps virus; TuV, Tuhoko virus; TiV virus, Tioman virus; MenV, Menangle virus; APMV, avian paramyxovirus; MeV, measles virus; PPRV, peste des petits ruminants virus; DoV, dolphin morbillivirus; PDV, phocine distemper virus; CDV, canine distemper virus; NaV, Nariva virus; MoV, Mossman virus; BtV, bat paramyxovirus; JV, J virus; BV, Beilong virus; NiV, Nipah virus; HeV, Hendra virus; TV, Tupaia paramyxovirus; BPIV, bovine parainfluenza virus; PPIV, swine parainfluenza virus; HPIV, human parainfluenza virus; SeV, Sendai virus; AsaPV, Atlantic salmon paramyxovirus; AnV, anaconda paramyxovirus; FDLV, Fer-de-Lance paramyxovirus.

identity between partial RdRp gene sequences from this study and SARS-related CoV circulating among rhinolophid bats in Europe (Fig. 1A and 3). Besides, we detected the first bat *Betacoronavirus 1* strains ($n = 5$) in *M. emarginatus* bats from 3 different colonies in 2015 (Bech-Kleinmacher) and 2016 (Bissen and Platen) (Fig. 1A; Table 1). All strains from Luxembourg were highly similar to each other and to *Betacoronavirus 1* strains identified in various mammalian species (>99% nucleotide identity between partial RdRp gene sequences) (Fig. 3 and 4).

Sequencing of the partial spike gene was attempted for all novel bat CoV strains but was successful only for the *Betacoronavirus 1* strains. As with the RdRp gene, all spike gene sequences were highly similar to each other and shared >98% nucleotide identity with the *Betacoronavirus 1* strains from other mammalian species (Fig. 4B).

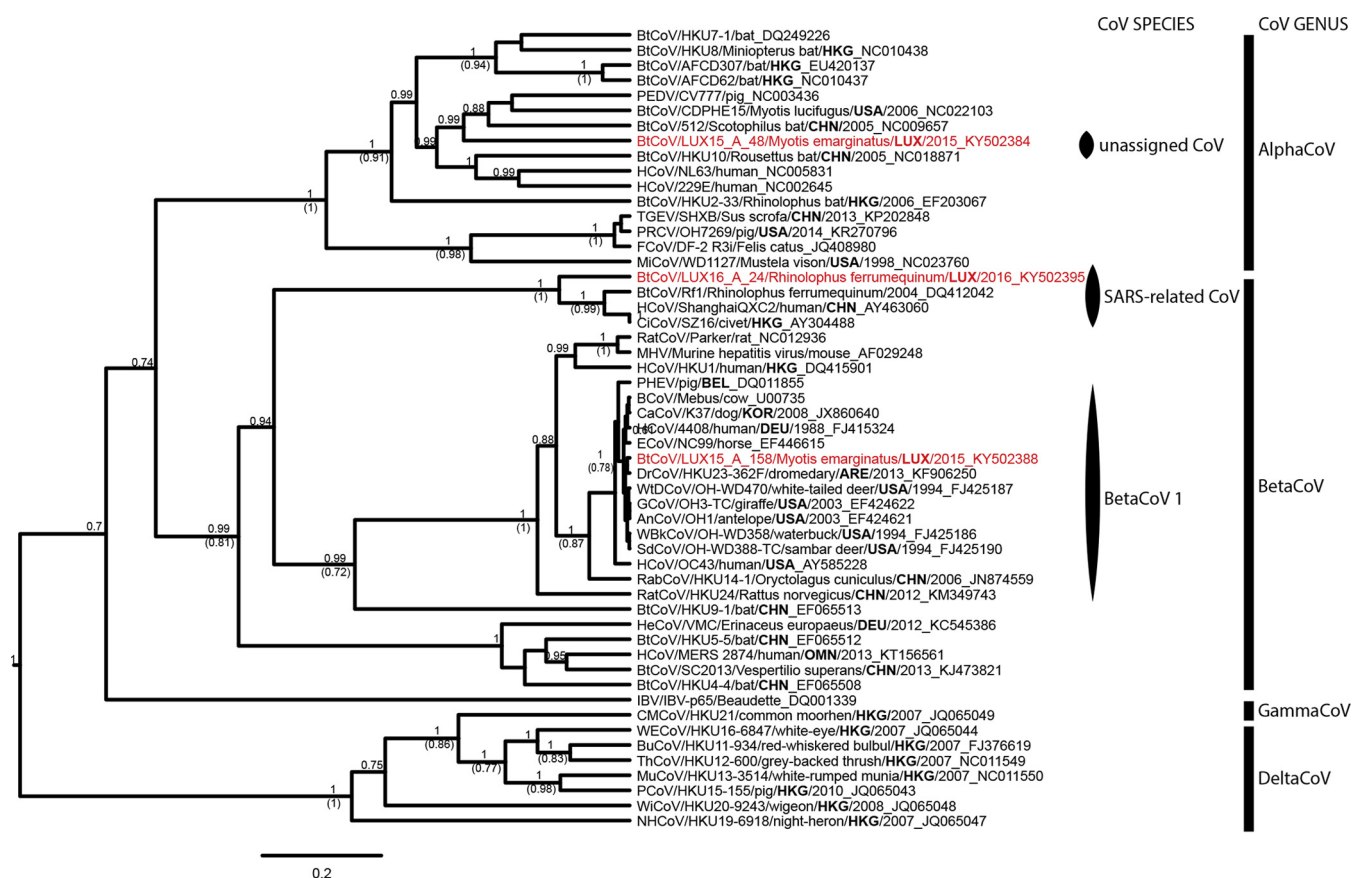
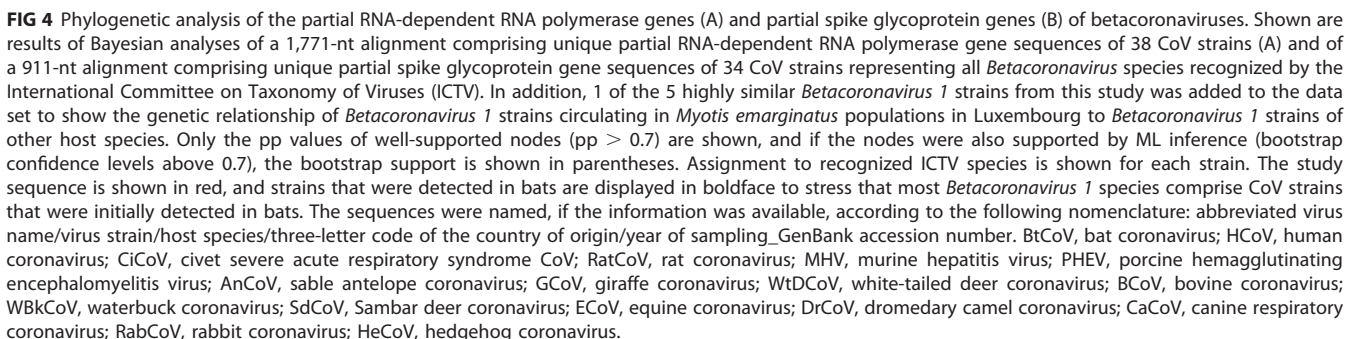


FIG 3 Phylogenetic analysis of the partial RNA-dependent RNA polymerase genes of all CoV genera. Shown are results of Bayesian analyses of an 853-nt alignment comprising unique partial RNA-dependent RNA polymerase gene sequences of 50 CoV strains representing all CoV species recognized by the International Committee on Taxonomy of Viruses (ICTV), as well as novel unassigned but putative CoV species. Three of the 43 CoV from this study were added to the data set to represent the genetic diversity of CoV circulating in *Myotis emarginatus* and *Rhinolophus ferrumequinum* populations in Luxembourg. The deltaCoV strains served as the outgroup for the phylogenetic analyses. Only the pp values of well-supported nodes (pp > 0.7) are shown, and if the nodes were also supported by ML inference (bootstrap confidence levels above 0.7), the bootstrap support is shown in parentheses. For each strain, the CoV genus assignment is shown. Assignment to recognized ICTV species is shown only for the study sequences displayed in red. A phylogenetic tree highlighting the CoV species of every strain can be found in the supplemental material (Fig. S3). The code for the country of origin of each strain is shown in boldface to stress the vast geographic spread of CoV. The sequences were named, if the information was available, according to the following nomenclature: abbreviated virus name/virus strain/host species/three-letter code of the country of origin/year of sampling_GenBank accession number. BtCoV, bat coronavirus; PEDV, porcine epidemic diarrhea virus; HCoV, human coronavirus; TGEV, transmissible gastroenteritis virus; PRCV, porcine respiratory coronavirus; FCoV, feline coronavirus; MiCoV, mink coronavirus; CiCoV, civet severe acute respiratory syndrome CoV; RatCoV, rat coronavirus; MHV, murine hepatitis virus; PHEV, porcine hemagglutinating encephalomyelitis virus; BCoV, bovine coronavirus; CaCoV, canine respiratory coronavirus; ECoV, equine coronavirus; DrCoV, dromedary camel coronavirus; WtDCoV, white-tailed deer coronavirus; GCoV, giraffe coronavirus; AnCoV, sable antelope coronavirus; WBkCoV, waterbuck coronavirus; SdCoV, Sambar deer coronavirus; RabCoV, rabbit coronavirus; HeCoV, hedgehog coronavirus; IBV, infectious bronchitis virus; CMCoV, common moorhen coronavirus; WECoV, wigeon coronavirus; BuCoV, bulbul coronavirus; ThCoV, thrush coronavirus; MuCoV, munia coronavirus; PCoV, porcine coronavirus; WiCoV, white-eye coronavirus; NHCov, night heron coronavirus.

DISCUSSION

Bats are natural reservoirs of numerous viruses with zoonotic potential. Of particular interest are CoV and PV, which share several traits allowing their adaptation to new ecological niches and hosts: high mutation rates, poor RNA proofreading capability, and genetic recombination (36–38). In line with previous studies (see, e.g., references 3, 4, and 23), we found genetically diverse CoV and PV strains in bats that are known to forage in and around human settlements in Luxembourg (Fig. 2 to 4; Table 1). Shedding rates may have been underestimated due to RNA degradation, low viral loads in feces (39), and the reduced sensitivity of degenerate primers. However, the sample collection and processing protocol was optimized to minimize the degradation of viral particles and of RNA, as well as inhibition. We acknowledge that the adenovirus control did not control for inhibition during the reverse transcription step. Although relatively susceptible to PCR inhibition and RNA degradation, fecal samples have been systematically



Plowright et al. proposed three scenarios to explain temporal variations in virus

shedding in bats: (i) virus reactivation in persistently infected bats, (ii) seasonal epidemic cycles aligning with the physiology of the bats' life cycle, or (iii) transient epidemics due to waning immunity (19, 46). In agreement with a previous study (4), we observed no temporal variation in PV shedding, possibly because of its low prevalence. In contrast, and in line with another study (40), a significant increase in *Alphacoronavirus* shedding was found in July, possibly due to periparturient stress (40, 47) (Fig. 1B).

The lack of similar reference sequences complicated the genetic and phylogenetic characterization of the virus strains detected. Nevertheless, we identified novel PV and *Alphacoronavirus* strains that are related to bat viruses from distant regions of the world (Fig. 2 and 3). Also, according to the PV species discrimination criterion published previously (amino acid distance in the L gene, >7 to 7.5% in the L gene) (4), the study sequences may represent putative novel PV strains, but this finding needs confirmation by whole-genome sequencing. Through amino acid sequence analysis of the partial RdRp gene, the topologies of the phylogenetic trees, and BLASTn analyses, the new CoV obtained in this study were found to be sufficiently divergent to represent a novel RdRp-based grouping unit (RGU) (5, 23). We found no evidence of interspecies transmission, although a mixed-species colony was monitored for 2 years (Fig. 1). Taken together, these findings confirm previous studies suggesting an association between *Alphacoronavirus* and host taxa rather than between geography and viral evolution, and thus close virus-host coevolution (23, 48–50).

On the other hand, the detection of highly similar virus strains in different colonies (Fig. 1A; Table 1) is indicative of a social link between *M. emarginatus* colonies in Luxembourg. This is of particular interest with respect to ongoing efforts for the conservation of this species. Indeed, short foraging distances (26) and lifelong roost fidelity complicate the preservation of *M. emarginatus* (51, 52). Since migratory distances of 35 to 126 km between summer and winter roosts have been reported (51, 52), and since all Luxembourgish colonies are within 45 km of each other (Fig. 1A), bats from different colonies may assemble during the autumn swarming of the males (32, 53). Thus, male bats may play a particular role in virus transmission, which warrants further investigation. A better understanding of the dynamics of bat-associated viruses may indirectly benefit these endangered species by providing information about foraging and mating behavior.

In contrast to the pattern of *Alphacoronavirus* evolution, host switching is a major evolutionary mechanism of *Betacoronavirus 1*. For instance, SARS-CoV and MERS-CoV circulated in bats before crossing the species barrier to infect an intermediate host, which, in turn, infected humans (8, 13, 54, 55). Bat SARS-CoV even use the same receptor for cell entry as their human counterparts, and they have been detected in rhinolophid bats (8), which also host genetically diverse SARS-related CoV (23, 56–59). Also in our study, *R. ferrumequinum* from Bech-Kleinmacher shed SARS-related CoV strains (Fig. 3). Although it is unlikely that these CoV represent a direct threat to humans, the potential risk of adaptation to the human host should not be ignored (60–62). The *Betacoronavirus 1* species is another exception to the typical host specificity of CoV. This species comprises highly similar viruses of distantly related mammals (6, 63–66), and so far, only a single, short *Betacoronavirus 1* sequence has been obtained from a bat (10). Most interestingly, we show here that *M. emarginatus* bats from different roosts shed *Betacoronavirus 1* strains (Fig. 1A) that are highly similar and closely related to *Betacoronavirus 1* strains detected in various other animal species (Fig. 4). Most-recent-common-ancestor analyses of *Betacoronavirus 1* suggested that the group appeared only recently and has low host specificity (67–69). For example, *Betacoronavirus 1* strains detected in exotic ruminants such as giraffes or antelopes are thought to represent spillover viruses of bovine CoV that underwent adaptive mutations (63, 65). Moreover, a possible animal origin of human CoV (HCoV) OC43 has been revealed by molecular clock analysis of the spike gene (68, 69), which provides an indication of host range and tissue tropism. The permissiveness of human cells to certain *Betacoronavirus 1* strains further underlines the potential of these strains to be transmitted across species (65, 67). Also in this study, all spike gene sequences were

highly similar to *Betacoronavirus 1* sequences from other mammalian species, reflecting the genetic stability typical of the lineage (63, 70, 71). To further investigate the role of bats as a reservoir of betacoronavirus 1, studies focusing on the host range of this CoV species are warranted.

In conclusion, we have shown that bats in Luxembourg, Western Europe, are hosts of novel virus strains that may be able to overcome the species barrier. *Betacoronavirus 1* strains with spike and RdRp genes genetically highly similar to those of mammalian strains were detected in synanthropic bats. In addition, we identified SARS-related CoV that may infect humans after a viral adaptation process (60–62). As shown before for bat lyssaviruses (34), our study highlights a certain risk for zoonotic transmission of bat viruses in particular, since the foraging and roosting sites of most indigenous bat species overlap with human and animal habitats. To mitigate this risk, it is important to monitor viruses circulating in synanthropic bats and putative intermediate hosts and to identify factors that affect bat populations.

MATERIALS AND METHODS

Samples. In 2015 and 2016, fecal samples ($n = 624$) were collected from a mixed *R. ferrumequinum*–*M. emarginatus* nursing colony in Bech-Kleinmacher, using a longitudinal approach. Samples were collected (i) after the resettling of the colony in the summer roost and before the birth of the juveniles (June 2015 [$n = 100$]; May 2016 [$n = 99$]), (ii) during lactation (July 2015 [$n = 126$]; June 2016 [$n = 111$]), and (iii) before the colony returned to the winter roost (September 2015 [$n = 100$]; September 2016 [$n = 88$]). In 2016, in the framework of a cross-sectional study, fecal samples ($n = 254$) were collected from 6 of the 14 synanthropic *M. emarginatus* colonies known in Luxembourg (Table 1; Fig. 1). At the beginning of June 2016 and before the birth of the juveniles, the population size of every known *M. emarginatus* maternity colony in Luxembourg (Table 1; Fig. 1A) was assessed by counting the bats emerging from the roost and/or the bats from a photograph taken in the roost, according to the *Guidelines for Surveillance and Monitoring of European Bats* (72).

The monitoring and sample collection were approved by the Ministry of Sustainable Development and Infrastructure Luxembourg (reference no. 86503 CG/ne).

Fresh feces were collected on a clean tarpaulin (left for 2 to 12 h underneath the roost) and were individually placed in 2-ml tubes using single-use spatulas. Samples were kept at $+4^{\circ}\text{C}$ during transport to the laboratory, where they were directly processed. The bat species was identified by visual inspection of the feces and of the bat cluster hanging above the collection site. Species identification was confirmed for virus-positive samples by sequencing of mitochondrial DNA (see below).

The study data set is described in Table 1, and the primer sequences can be found in Table 2.

Nucleic acid extraction. Entire bat droppings (approximately the size of a long grain of rice) were individually resuspended in 1 ml of prechilled virus transport medium (prepared according to the WHO protocol [73]) and were homogenized using stainless steel beads (Qiagen, Venlo, The Netherlands) and a TissueLyser II system (Qiagen).

After centrifugation at $2,200 \times g$ for 20 min, the supernatant was transferred to a new 2-ml tube and was stored at -80°C until further processing. Before nucleic acid extraction, each sample was centrifuged at $2,200 \times g$ for 10 min and was spiked with an extraction control (i.e., human adenovirus C5). Concurrent extraction of DNA and RNA was performed with the QIAamp viral RNA minikit (Qiagen) according to the manufacturer's protocol. To test for inhibition and to confirm successful extraction, each sample was tested using a real-time PCR specific for adenovirus (74).

Virus detection. All samples were tested for CoV and PV by reverse transcription-PCRs (RT-PCRs) with degenerate primers in a nested format. The PCRs were performed in a final volume of 25 μl . In the first step of the nested PCR, the Qiagen One-Step RT-PCR kit (Qiagen) was used. The CoV PCR master mix contained 2 μl of RNA, 1 μM each primer, 1 mM MgCl_2 , and 1 mM each deoxynucleoside triphosphate (dNTP), and the PV PCR master mix contained 250 nM each primer, 1.5 mM MgCl_2 , and 100 μM each dNTP. In the second step of the nested PCRs, the CoV PCR master mix contained 2.5 μl of 1:5-diluted PCR product, 700 nM each primer, 2 mM MgCl_2 , and 200 μM each dNTP, whereas the PV PCR master mix contained 0.1 μl of undiluted PCR product, 600 nM each primer, 2 mM MgCl_2 , and 200 μM each dNTP. The adenovirus detection PCR was similar to the CoV PCR, but 2.5 μl of DNA was used and 560 nM probe was added to the mix. In the second step of the nested PCRs, in the adenovirus detection PCR, and in the bat species identification PCR, the Platinum *Taq* DNA polymerase kit (Life Technologies Europe B.V., Ghent, Belgium) was used. The CoV primers target the RNA-dependent RNA polymerase (RdRp) (modified from reference 75), whereas the PV primers target the L genes (76) of all known strains of the respective viral families. An avian infectious bronchitis virus (an avian CoV) and a measles virus (a human PV) served as positive controls in the CoV and PV PCRs. Details about the primers can be found in Table 2.

Sequencing. PCR-positive samples were identified by agarose gel electrophoresis. Where multiple bands were present, amplicons of the appropriate size were excised from the gel and were purified with the QIAquick gel extraction kit (Qiagen). PCR products giving a single band in the gel electrophoresis were directly purified using the JetQuick extraction kit (GenoMed, Löhne, Germany). Sequencing was performed using the BigDye Terminator kit (Applied Biosystems, Foster City, CA), run on an ABI 3130

TABLE 2 Primers used for detection and sequencing of coronaviruses and paramyxoviruses

Virus	Target protein	Primer sense	Primer sequence (5'–3')	Amplicon size (bp)	Reference
Detection					
<i>Paramyxoviridae</i>	L protein, subunit of RNA-dependent RNA polymerase	Forward Reverse	GAAGGATTTGTCAIAARNTNTGGAC GCTGAAGTTACGGTTCICCDATRTTNC	660	76
<i>Coronaviridae</i>	Replicase polyprotein 1ab	Forward Reverse	GTGTCTCAATGGTTTCARGNGAYAA GGTGGGAYTAYCCKAARTG	580 602	75
		Forward Reverse	TGYTGSWRCARAAAYTCRTG GGTTGGACTATCCTAAGTGTGA	555	This study
Human adenovirus	Hexon gene	Reverse nested PCR Forward Reverse	CCAACAYTTNGARTCWGCCAT GCCACSGTGGGTTYCTAAACTT GCCSAGTGGKCDTACATGCACATC	130	This study This study
		Probe	FAM-TGCCACAGACCCGGCTCAGGTACTCCGA-TAMRA	74	74
Sequencing					
<i>Alphacoronavirus</i>	Replicase polyprotein 1ab	Forward Reverse	TGTGAAGGCCTTACAGCGTC AGAGCCACAWACACACACA	670	This study
	Replicase polyprotein 1ab	Forward Reverse	TGATGCAGCTGYARAGACTTC CCAGAAGTCGTACCCACGAG	690	This study
<i>Betacoronavirus 1</i>	Replicase polyprotein 1ab	Forward Reverse	AGACATCGTCCCATCCATC AGCTACACGTGGTGTTCCTG	729	This study
	Replicase polyprotein 1ab	Forward Reverse	CATATCATCCAGCCGCCAT TGCTGTTTGTAGTTGCGGC	584	This study
	Replicase polyprotein 1ab	Forward Reverse	CCGTTGTTATAGCCGCAAC AGCGCTACTGAGTTTGACAG	613	This study
	Spike gene	Forward Reverse	GTGAGCACTGTTCCGGTCTT AGCAATGCTGGTTCGGAAGA	432	This study
	Spike gene	Forward Reverse	ATGGCATTTGGATACAG TAATGGAGAGGGCACCCGACTT	492	65
	Spike gene	Forward Reverse	GGGTTACACCTCTCACTCT GCAGGACAAAGTGCCTATACC	767	65
SARS-related coronavirus	Replicase polyprotein 1ab	Forward Reverse	AGTTGAGGTGGTCGACAAAGT GCAGTGGTAGCATCTCTGA	650	This study
Bat species identification	Cytochrome <i>b</i>	Forward Reverse	ATGACCAACATTCGMAARTCYCAC TGATGACGGTTGCTCCTCA	390	This study

sequencer (Applied Biosystems). Partial L gene sequences of PV were obtained using the detection primers. Partial sequencing of CoV was attempted using specific primers targeting the conserved RdRp gene, as well as the spike glycoprotein gene. To reliably identify the bat species of all virus-positive samples, partial cytochrome *b* sequences were obtained. The bat species identification PCR was performed using the Platinum *Taq* DNA polymerase kit (Life Technologies Europe B.V.) in a final volume of 25 μ l containing 5 μ l of DNA, 700 nM each primer, 4 mM $MgCl_2$, and 400 μ M each dNTP. New primer sets were designed and evaluated with Geneious software (version 7.1.7; Biomatters Limited, Auckland, New Zealand) and Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>). Details about the sequencing primers can be found in Table 2.

Sequence and phylogenetic analyses. Sequence assembly and processing were performed in Geneious, version 7.1.9 (77). A BLASTn search against the sequences in GenBank (<https://www.ncbi.nlm.nih.gov/GenBank/>) was performed with the default parameters. Phylogenetic trees based on nucleotide sequences of the partial RdRp and spike genes for CoV, and of the partial L gene for PV, were constructed. In order to increase the phylogenetic resolution and because of the high genetic similarity of the virus strains obtained, only the longest sequences of high quality were selected among the novel sequences and were aligned with representative GenBank sequences using the ClustalW algorithm (78), as implemented in Geneious. Phylogenetic trees based on all study sequences of good quality are shown in the supplemental material (Fig. S1 and S3). Poorly aligned positions in the alignments were eliminated using Gblocks (79) as implemented in Seaview, version 4 (80). Maximum likelihood (ML) and Bayesian inference of evolution were estimated in PhyML (81, 82) and BEAST (83, 84), respectively. The best substitution model identified by jModelTest (85) according to the Bayesian information criterion (BIC) and/or Akaike information criterion (AIC) values was used. A bootstrap test including 1,000 replicates was performed for each ML tree. For the Bayesian Markov chain Monte Carlo (MCMC) approach, the parametric model “Constant Size” was used as the prior, and the analyses were performed with a lognormal relaxed clock. The MCMC run was at least 2×10^7 steps long, with sampling every 10^3 steps. Convergence was assessed on the basis of the effective sampling size using Tracer, version 1.6 (86). The results of the Bayesian phylogenetic inference were summarized in a maximum clade credibility tree using the Tree Annotator program after a 10% burn-in. Tree topology was tested by posterior probability (pp), and only the pp values of well-supported nodes (pp > 0.7) are shown in the figures. Since the topologies of the trees based on Bayesian and ML inference largely overlapped, only the maximum clade credibility trees are shown. However, for the nodes also supported by ML inference (bootstrap confidence levels above 0.7), the bootstrap support is shown in parentheses in the figures. The scale bar for each tree indicates the average number of nucleotide substitutions per site (Fig. 2 to 4).

Statistical analyses. Statistical analyses were performed in R software (version 3.1.0.; R Foundation for Statistical Computing, Vienna, Austria [<https://www.r-project.org/>]) (24). Logistic regression was performed to predict the binary outcome (i.e., the presence or absence of detectable *Alphacoronavirus* shedding by *M. emarginatus*) based on the categorical predictor “season” with the levels “May/June,” “July,” and “September” and using a logistic function.

Accession number(s). The viral and mitochondrial sequences obtained in this study have been submitted to GenBank under accession numbers KY502383 to KY502414, as well as KY707827 and MF048874 to MF048903.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01326-17>.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

ACKNOWLEDGMENTS

We thank the many people who contributed significantly to the success of this study by providing logistical support or by supervising or performing the sample collection. In this context, we mention in particular the investigators of a research project on the genetics of *M. emarginatus* cofinanced by the Ministry of Sustainable Development and Infrastructure (MDDI, Environment Department) and the Natural History Museum of Luxembourg: Simone Schneider and Mara Lang of the Biological Station (SICONA, Luxembourg), as well as Alain Frantz of the Centre de Recherche Scientifique (Musée National d'Histoire Naturelle, Luxembourg). We are also grateful to the owners of the buildings containing the bat roosting sites for approving this study, as well to Martyna Marynowska and Claire Dording for performing part of the laboratory analyses.

This study was funded by the Ministry of Foreign and European Affairs, Luxembourg (project “Microbiology for development IV”), which was not involved in study design, data collection and interpretation, or the decision to submit the work for publication. We declare that we have no conflict of interest relevant to the study.

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